(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: **0 037 583**
A1

(12) # EUROPEAN PATENT APPLICATION

(21) Application number: **81102650.9**

(22) Date of filing: **08.04.81**

(51) Int. Cl.³: **C 12 Q 1/00**
C 12 Q 1/34, C 12 Q 1/44

(30) Priority: **09.04.80 FI 801117**
18.04.80 FI 801258
27.02.81 FI 810616

(43) Date of publication of application:
14.10.81 Bulletin 81/41

(84) Designated Contracting States:
AT BE CH DE FR GB IT LI LU NL SE

(71) Applicant: **KSV-Chemicals Oy**
Hösmärinkuja 4 D 15
SF-02760 Espoo 76(FI)

(72) Inventor: **Kinnunen, Paavo K. J.**
Punarinnantie 4
SF-02660 Espoo 66(FI)

(72) Inventor: **Schröder, Tom M.**
Hösmärinkuja 4 D 15
SF-02760 Espoo 76(FI)

(72) Inventor: **Virtanen, Jorma A.**
Ristiaallokonkatu 4 C 71
SF-02320 Espoo 32(FI)

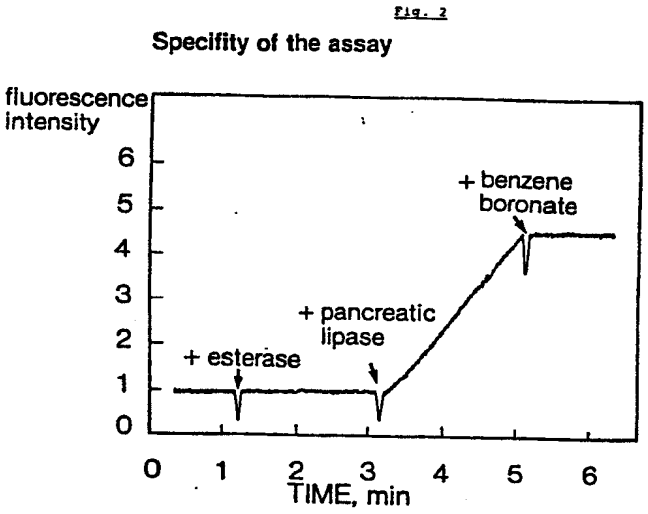
(74) Representative: **Brandes, Jürgen, Dr.**
Dipl.-Chem. et al,
Patentanwälte H. Bartels, Dipl.-Chem. Dr. Brandes
Dr.-Ing. Held, Dipl.-Phys. Wolff Thierschstrasse 8
D-8000 München 22(DE)

(54) **Method of fluorometrically measuring the activity of fat-degrading enzymes and means for carrying out the method.**

(57) The object of the present invention is a new method for fluorometrically assaying the activity of fat-degrading enzymes, such as lipases and phospholipases in samples containing said enzyme, such as in serum. According to the method the enzyme containing sample is reacted with a substrate containing acyl- or alkylglycerols or -phosphoglycerols having at least one fluorescent group, such as a pyrene group. The compounds may in addition contain fluorescence quenching groups. The enzyme hydrolyzes the substrate thus giving rise to changes in the fluorescence intensity during the enzyme reaction and the changes in the intensity are measured at a specific emission wavelength of the fluorescent group employed. The rate of change of the invensity is proportional to the enzyme activity in the sample.

The invention concerns also the new compounds containing fluorescent groups to be used in the method.

Fig. 2



EP 0 037 583 A1

Method of fluorometrically measuring the activity of fat-degrading enzymes and means for carrying out the method

The object of the present invention is a clinico-analytical assay method which is based on changes occurring in the fluorescence intensity. One such method is lipase and phospholipase, especially phospholipase A_2 assay in serum. Lipases are enzymes which split triacyl-glycerols into free fatty acids, glycerides and glycerol. Lipases differ from each other i.a. as to their stereospecificity, and some lipases have the ability to distinguish between the fatty acids in the 1- and 3-positions of an sn-triacyl-glycerol. Phospholipase A_2 splits the fatty acid in the 2-position with respect to the phosphoryl group of a phospholipid, the end products being a lysophospholipid and a free fatty acid.

Important from the standpoint of clinical chemistry are the lipase and phospholipase enzymes which under normal conditions are released into the gastrc-intestinal tract. I.a. in connection with pancreatitis these enzymes, however, are released into the blood stream and consequently it is of diagnostical importance to measure the enzyme activity in the plasma.

Hitherto two methods for measuring pancreatic lipase in serum have been used. According to the first method, serum is reacted with a triglyceride emulsion whereafter the liberated fatty acids are measured, usually titrimetrically. This method is cumbersome, inexact and its reproducibility poor. Another method used is the nephelometric. According to this method the decrease in the scattering of light as a result of the degradation of the emulsion particles by the lipase is measured, as a fuction of time. This method is relatively sensitive but not very reproducible. Also the endogenic triglycerides of the serum interfere very strongly with the measurement

and it is not possible to make nephelometrically reliable lipase assays from lipemic samples.

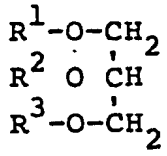
The assay methods for phospholipases have been based on the use of a radioactive substrate, whereby e.g. ^{14}C- or ^{3}H-atoms are introduced into the fatty acid in the 2-position and the degree of radioactivity of the fatty acid split by the phospholipase A_2 is measured. The method is however cumbersome because of the many stages involved, and it requires special apparatuses, i.a. a scintillation counter.

The object of the present invention is to provide a fluorometric assay method wherein substrates containing glycerol and phosphoglycerol compounds are used, into which compounds fluorescent groups have been introduced, and optionally also quencher groups, and wherein the changes in the fluorescence intensity due to the enzymatic reaction are measured. By means of the method it is possible, without any cumbersome separation steps, to determine for example the lipase and phospholipase activity in a serum sample, which makes the method especially well suited for hospital laboratory use.

The object of the present invention is thus a method for fluorometrically measuring the activity of fat-degrading enzymes in samples containing said enzyme according to which method the enzyme containing sample is combined with a substrate which contains an acyl- or an acyl-alkyl-glycerol or -phosphoglycerol which reacts with the enzyme to be assayed, wherein at least one of the acyl or alkyl groups contains a fluorescent group and the other groups may optionally contain a fluorescence quenching group, the substrate is excited at the specific excitation wave-length of the fluorescent group in question and the change, due to the enzyme, in the fluorescence intensity of the substrate per time unit is measured at a specific emission wave length of the fluorescent group, the rate of change being directly proportional to the enzyme

activity in the sample.

In the method according to the invention compounds are preferably used having the formula

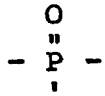


I

wherein

a) at least two of the groups R^1, R^2 and R^3 denote a saturated or unsaturated acyl group with 3 to 36 carbon atoms, and the third can denote also hydrogen or a saturated or unsaturated alkyl group with 3 to 36 carbon atoms, or

b) one of the groups R^2 and R^3 denotes a phosphoryl group



- OR, wherein R is hydrogen, ethanolamin, ethylene glycol, choline, glycerol or serine, and the other, as well as R^1, denotes an acyl or alkyl group as defined above, provided that the carbon atom in the 2-position to the phosphoryl group may contain only an acyl group, and

wherein in the compound of formula I always at least one of the acyl or alkyl groups R^1, R^2 and R^3 is substituted with a fluorescent group, and wherein one or both the other groups may be optionally substituted with a fluorescence quenching group.

The fluorescent group may be pyrene, tetracene, anthracene, phenanthrene, naphthalene, coumarone, coumarin, acridine, benzocarbazone, aminonaphtalenesulfonic acid, mono-, di- or tri-iodo-benzene, perylene, phenyloxadiazole, diphenyloxazole, alloxazine, stilbene, dibenzofuran, fluorene, fluorenone, oxopiperazine, p-quinone, methylumbelliferone, phenazine, phenyl-indole, quinoline, di-ethylaniline, phenol, diphenylacetylene, benzotiophen, pyrimidine, xanthone, thiocarbocyanide, 1,3,5,7-deka-

tetra-ene.

A suitable fluorescent group is pyrene because of its well-characterized fluorescence behaviour (Th. Förster, Angew. Chem. 81, 364 (1969) and S.C. Charlton et al, The Journal of Biol. Chem. Vol. 251, No 24, 7952 (1976)).

The fluorescence may be sensitized when using compounds of the formula I containing two fluorescent groups, by introducing into one of them electron donating groups such as methyl, methoxy, hydroxyl or dimethylamino groups, and into the other electron attracting groups, such as cyano and nitro groups.

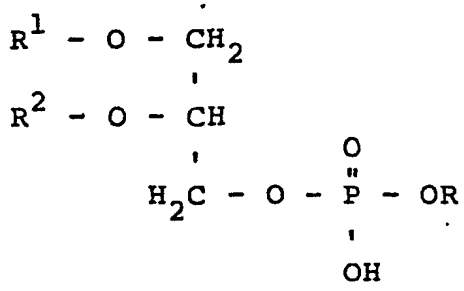
As a quencher preferably halogen is used, such as bromine, iodine or chlorine, or halogen substituted groups, such as halogen substituted phenyl groups.

A first sub group a) of the compounds of the formula I comprises for lipase assay suitable triacyl-, diacyl-monoalkyl- and diacyl-glycerols, respectively, wherein at least one of the acyl and alkyl groups is substituted with a fluorescent group, and wherein one other or both the other groups may be substituted with a fluorescence quenching group.

Of these may be mentioned especially the triacyl-glycerols or the formula I wherein one, two or all three acyl groups may contain one, two or three fluorescent groups, suitably pyrene groups, as well as 1,3-diacyl-2-alkyl-sn-glycerols, which can be substituted with fluorescent groups as the triacyl-glycerols.

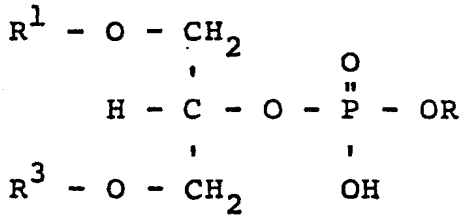
Usable intramolecularly quenched compounds of this group are for example triacyl- or 1,3-diacyl-2-alkyl-sn-glycerols which in their 2-position contain a fluorescent group, whereby the quenching group, suitably a bromine group, is in the 1- and/or 3-position.

The second sub group b) comprises phospholipid compounds suitable for phospholipase A_2 assay and having the formula



Ia

and



Ib

wherein in the formula Ia the group R^2 and in the formula Ib the group R^1 is an afore mentioned acyl group, and wherein at least one of the groups R^1 and R^2, or R^1 and R^3, respectively, contains a fluorescent group, and the other optionally a quenching group.

Of these may be mentioned especially the 1,2-diacyl- or 1-alkyl-2-acyl-compounds of formula Ia, wherein one or both of the groups in the positions 1 and 2 contains a fluorescent group. A suitable compound is also the corresponding compound wherein the fluorescent group has been quenched with a bromine or iodine atom or with some other halogen containing group.

Especially advantageous compounds are the following

I. 1-oleoyl-2-/4-(3-pyrenyl)-buturoyl/-3-oleoyl-sn-glycerol

II. 1-(6-bromohexanoyl)-2-/4-(3-pyrenyl)-buturoyl/-3-oleoyl-sn-glycerol

III. 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-3-oleoyl-sn-glycerol

IV. 1-(4-(2-anthroyl)-buturoyl)-2-(6-bromohexanoyl)-3-oleoyl-sn-glycerol

V. 1,2-di-(4-(4-pyrenyl)-buturoyl)-sn-glycero-sn-3-phosphorylglycerol

VI. 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycero-3-phosphorylglycerol

VII. 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycero-3-phosphorylethylenglycol

VII. 1-(hexatriacontanyl)-2-(4-(3-pyrenyl)-buturoyl)-sn-glycero-3-phosphorylglycerol

IX. 1-(6-bromohexanoyl)-2-(4-(3-pyrenyl)-buturoyl)-sn-glycero-3-phosphorylcholine

X. 1-(4-(2-anthroyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycero-phosphorylglycerol

XI. 1-(10-(2-anthroyl)-decanoyl)-2-(10-(3,5-dibrom-4-methoxifenyl)-decanoyl)-sn-glycero-phosphoryl-glycerol

XII. 1-(10-(2-anthroyl)-decanoyl)-3-linoleyl-sn-glycerol-2-phosphorylcholine

XIII. 1-(10-(3-pyrenyl)-decanoyl)-3-(12-bromododecanoyl)-sn-glycerol-2-phosphorylcholine

XIV. 1,2-di-(4-(3-pyrenyl)-buturoyl)-sn-glycerol

XV. 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycerol

XVI. 1-(6-bromohexanoyl)-2-(4-(3-pyrenyl)-buturoyl)-sn-glycerol

When in the method a compound is used which contains a single fluorescent group, such a compound forms when emulsified in oil, whereby the individual molecules are

packed close together, a so-called intermolecular dimer, i.e. an excimer, which, when excited at the excitation wavelength of the fluorescent group in question, fluoresces at the excimer wavelength characteristic for this group. When a substrate containing such a compound is reacted with an enzyme, one obtains through hydrolysis, depending on the enzyme and the compound used, as degradation products free fatty acids as well as mono- or disubstituted glycerols or lysophospholipides, respectively, which leave the emulsion particles whereby the intermolecular interaction between the fluorescent groups disappears and consequently the excimer fluorescence intensity decreases and the intensity of the monomer fluorescence, due to the fluorescent free fatty acid or the fluorescent glycerol product, increases.

For example, the above mentioned compound I, 1-oleoyl-2-(4-(3-pyrenyl)-buturoyl)-3-oleoyl-sn-glycerol, forms emulsified in oil, an intermolecular excimer, which, when excited at the excitation wavelength of about 320-345 nm, fluoresces at the excimer wavelength of 470 nm of pyrene. When such an emulsion reacts with pancreatic lipase there is formed, because the pancreatic lipase does not exhibit stereospecificity, two free fatty acids and 2-(4-(3-pyrenyl)-buturoyl)-glycerol. As a result of the enzymatic reaction the excimer fluorescence intensity decreases and that of the monomer fluorescence correspondingly increases. The change in fluorescence intensity may be followed either at the excimer wavelength of pyrene of ca 470 nm or at its monomer wavelength of ca 390 to 400 nm, and the rate of change of the intensity is directly proportional to the amount of fluorescent compound degraded by the enzyme, i.e. to the enzymatic activity.

A strongly fluorescent intramolecular excimer is formed by compounds of the formula I which in the same molecule contain two or three fluorescent groups. As a result of the enzymatic reaction the interaction between

these fluorescent groups gradually disappears, whereby the excimer fluorescence intensity decreases and correspondingly the monomer fluorescence intensity increases. When using for example in the substrate the compound V, 1,2-di-(4-(3-pyrenyl)-buturoyl)-sn-glycero-sn-3-phosphorylglycerol, the phospholipase A_2 hydrolyzes the fatty acid chain in the 2-position and as reaction products a fluorescent free fatty acid and a fluorescent pyrene-fatty acid phosphatide are formed. As a result of the enzymatic reaction the pyrene excimer fluorescence at the wavelength of about 470 nm weakens and its monomer fluorescence at the wavelength of about 400 nm increases, and the rate of change of the fluorescence intensity is proportional to the degree of hydrolyzis. By following for example at the wavelength of about 470 nm the rate of change of the fluorescence intensity, it is possible to determine the amount of fluorescent compound degraded per time unit, which in turn is dependent on the enzymatic activity.

The enzymatic activity can be measured also by using according to the invention a compound which contains both a fluorescent as well as a group, or groups, preferably bromine atoms, quenching the intramolecular fluorescence. As a result of the enzymatic reaction a fatty acid containing either a fluorescent group or a quenching group is split off and a fluorescent fatty acid or a fluorescent glycerol compound is formed which fluoresces at the monomer wavelength. The increase in intensity, due to a lesser degree of quenching, is enhanced by the same phenomenon as when using a compound containing only one fluorescent group. The method may be carried out also by using an oil-emulsified substrate containing both a compound having one fluorescent group and a compound which contains a fluorescence quenching group. In oil these compounds are forced into close contact thus forming a so-called intramolecularly quenched macromolecule, which as a result of the enzymatic reaction is broken down into products which

fluoresce at the monomer wavelength.

In all cases the method is calibrated with solutions containing known amounts of fluorescent compound.

An object of the invention are also the compounds usable for carrying out the method according to the invention, especially the compounds of formula I, as new substances.

These compounds may be prepared e.g. by introducing into glycerol or D-mannitol the desired acyl and alkyl substituents, optionally containing fluorescent or quencher groups. The substituted D-mannitol is then split and reduced to the corresponding glycerol. A substituted glycerol thus obtained may be further substituted in its free position with a phosphoryl group or a derivative thereof to obtain the desired compound.

The following examples illustrate the invention.

Example 1

Phospholipase A_2 activity was measured using the following phospholipid substrate which in 2.90 ml contains

1 mM dioleoylphosphatidyl-glycerol
1 nM 1,2-di-(4-(3-pyrenyl)-buturoyl)-sn-glycero-sn-3-phosphorylglycerol
2 mM $CaCl_2$
2 mM cholate
50 mM tris-HCl, pH 7.4.

The fluorescence was measured with a Perkin-Elmer-fluorescense-spektrofotometer, excitation opening 3 nm, emission opening 6 nm, excitation wavelength 320 nm, emission wavelength 470 nm. The base level was determined whereafter 100 µl of a sample containing phospholipase A_2 from cobra venom was added to the substrate. The decrease in fluorescence intensity was followed as a function of time at the excimer wavelength of 470 nm using a recorder. For example, a decrease of 10 % in the fluorescence intensity in 10 minutes means that 100 µl of a sample contains phospholipase A_2 enough to hydrolyze 10 % of the total phospholipid content, i.e. about 0,1 mM = 100 µM phospholipid in 10 minutes, and thus the activity of the sample corresponds to 100 µM liberated fatty acid/minute and ml.

By changing the sensitivity level of the fluorometer it is possible to measure the degree of hydrolyzis in samples the activity of which varies between 1nM and

100 µM free fatty acid/minute and ml. When the results were compared with the results obtained by using a radioactive substrate, the result shown in Fig. 1 was obtained, from which it can be seen that the results obtained with both methods are uniform.

Example 2

The lipase activity was measured using the following lipid substrate

1.00 ml of a solution containing

50 µl olive oil

5 µl ethanol (=0.5 %)

1 µg 1-oleoyl-2-(4-(3-pyrenyl)-buturoyl)-3-oleoyl-sn-glycerol

5 mM Na-deoxycholate

50 mM tris-HCl, pH 8.4.

The fluorescence was measured using a fluorescence spectrofotometer connected to a recorder, excitation opening 20 nm, emission opening 20 nm, excitation wavelength 343 nm. Emission was folled at the wavelength 400 nm. First the base level was measured whereafter 50 µl of a sample containing lipase was added and the increase in fluorescence intensity per time unit was followed at the monomer wavelength of ca 400 nm.

In the appended Fig. 2 is shown an assay with normal and pathological serum. The fluorescence intensity Im was measured at the monomer wavelength 400 nm as a function of time. As fluorescent compound the above 1-oleoyl-2-(4-(3-pyrenyl)-buturoyl-3-oleoyl-sn-glycerol was used. Benzene boronate was used for stopping the a reaction.

Example 2 may be repeated but using as an intramolecularly quenched compound 1 µg of 1-(6-bromohexanoyl)-2-(4-(3-pyrenyl)-buturoyl)-3-oleoyl-sn-glycerol and measuring the increase in fluorescence intensity per time unit, as above.

Instead of an intramolecularly quenched compound one can use in the same assaying system 1 µg of 1-oleoyl-2-(4-(3-pyrenyl)-buturoyl)-3-oleoyl-sn-glycerol together with 1 µg of 1-oleoyl-2-(6-bromohexanoyl)-3-oleoyl-sn-glycerol, which in oil forms an intramolecularly quenched macromolecule. The increase in fluorescence intensity per time unit after the addition of enzyme is measured as above.

Example 3

The phospholipase A_2 activity was measured using a substrate which in 2.0 ml contains

0.5 mM $CaCl_2$
0.1 mM egglecithin
0.25 mM Na-deoxycholate
0.2 mM cholate
0.1 % (w/vol) bovine serum albumin
50 mM tris-HCl, pH 7.0
40 µg of fluorescent phospholipid.

As phospholipids the following compounds may be used:

a) 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycero-3-phosphorylglycerol
b) 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycero-phosphorylethylenglycol
c) 1-(hexatriacontanyl)-2-(4-(3-pyrenyl)-buturoyl)-sn-glycero-3-phosphorylglycerol
d) 1-(6-bromohexanoyl)-2-(4-(3-pyrenyl)-buturoyl)-sn-glycero-3-phosphorylcholine
e) 1-(4-(2-anthroyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycero-3-phosphorylglycerol
f) 1-(10-(2-anthroyl)-decanoyl)-2-(10-(3,5-dibromo-4-methoxyphenyl)-decanoyl)-sn-glycero-3-phosphoryl-glycerol
g) 1-(10-(2-anthroyl)-decanoyl)-3-linoleyl-sn-glycerol-2-phosphorylcholine

h) 1-(10-(3-pyrenyl)-decanoyl)-3-(12-bromododecanoyl)-sn-glycerol-2-phosphorylcholine

The lecithin and the fluorescent compound were dried solvent free in a nitrogen stream. Thereafter the Na-deoxycholate was added and the mixture sonicated using a Branson sonifier equipped with microtip at setting 4. Thereafter the bovine serum albumin was added, dissolved in buffer. The substrate was stable for several day provided 0.1 mM NaN_3 was added to prevent microbial growth.

To 2.0 ml of substrate 200 µl of serum is added. After mixing, the solution is transferred to a cuvette. Stirring is not necessary when emulsified substrates are used.

Fluorescence changes were measured using a Kontron SFM-23 spectrofluorometer equipped with a magnetically stirred cell (1.0 x 1.0 x 0.5 cm). Temperature was controlled with a cryostat at 37°C throughout the measurement. The fluorescence intensity signal was fed into a recorder. Excitation wavelength was 343 nm for the pyrenyl containing compounds and 370 nm for the anthroyl containing compounds, and the changes in the monomer fluorescence intensity were followed 400 nm for pyrenyl and at 450 nm for anthroyl.

Example 4

The lipase activity was measured using a substrate which in 2.0 ml contains

0.005 mM tributyrin
0.15 M NaCl
0.03 % (vol./vol.) Span 80)
0.01 % Tween 80) detergents
50 mM tris-HCl, pH 8.4
4.35 µg of fluorescent acylglycerol

a) 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-3-oleoyl-sn-glycerol

b) 1-(4-(3-anthroyl)-buturoyl)-2-(6-bromohexanoyl)-3-oleoyl-sn-glycerol

c) 1-(4-(3-pyrenyl)-buturoyl)-2-(6-bromohexanoyl)-sn-glycerol.

The lipid to be tested was dried in a stream of nitrogen whereafter the tributyrin and detergents were added, as well as the buffer solution, whereafter the mixture was sonified as in Example 3. In case the sample a was to be stored 0.1 mM of NaN_3 was added to prevent microbial growth. The substrate was stable for several days. The solution is to be thoroughly stirred prior to use.

To this solution 200 µl of serum was added, whereafter the mixture was transferred to a cuvette and the change in fluorescence intensity measured as stated in Example 3.

Fig. 1 Degradation of radioactive (A) and fluorescent (B) phospholipid in a phospholipase A_2 reaction. Enz. unit = enzymatic activity, nanomoles lysophospholipid per minute and ml. The amount of enzyme used was varied and the enzymatic activity measured with both methods.

Claims:

1. Method of fluorometrically measuring the activity of fat-degrading enzymes in samples containing said enzyme, c h a r a c t e r i z e d in that the sample containing said enzyme is combined with a substrate which contains a an acyl- or an acyl-alkyl-glycerol or -phosphorylglycerol which reacts with the enzyme to be assayed, wherein at least one of the acyl or alkyl groups contains a fluoroscent group and the other groups may optionally contain a fluorescence quenching group, the subtrate is excited at the specific excitation wave-length of the fluorescent group in question and the change, due to the enzyme, in the fluorescence intensity of the substrate per time unit is measured at a specific emission wave-length of the fluorescent group, the rate of change being directly proportional to the enzyme activity in the sample.

2. Method according to Claim 1, c h a r a c t e r i z e d in that a compound is used having the formula

$$\begin{array}{l} R^1\text{-O-CH}_2 \\ R^2\text{-O-CH} \\ R^3\text{-O-CH}_2 \end{array} \qquad \text{I}$$

wherein

a) at least two of the groups R^1, R^2 and R^3 denote a saturated or unsaturated acylgroup with 3 to 36 carbon atoms, and the third of these can denote also hydrogen or a saturated or unsaturated alkyl group with 3 to 36 carbon atoms, or

b) one of the groups R^2 and R^3 denotes a phosphoryl group $-\overset{\overset{O}{\|}}{\underset{\underset{OH}{|}}{P}}-OR$, wherein R is hydrogen, ethanolamine, ethylene glycol, choline, glycerol or serine, and the other, as well as R^1, denotes an acyl or alkyl group as defined

above provided, however, that the carbon atom in 2-position to the phosphoryl group may contain only an acyl group, and

wherein in the compound of formula I, always at least one of the acyl or alkyl groups R^1, R^2 and R^3 is substituted with a fluorescent group, and one or both other groups may be optionally substituted with a fluorescence quenching group.

3. Method according to Claim 1 or 2, characterized in that as a fluorescent group pyrene is used, whereby the substrate is excited at a wavelength of about 320 to 345 nm, and the rate of change of the fluorescence intensity is measured at either the monomer wavelength of pyrene of about 390 to 400 nm or at its excimer wavelength of about 470 nm.

4. Method according to any one of the Claims 1-3, characterized in that as a fluorescence quencher halogen, such as bromine or iodine, is used.

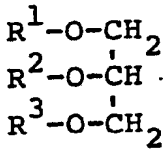
5. Method according to any one of the Claims 1-3, characterized in that when using a compound containing two fluorescent groups, the fluorescence is sensitized by introducing into one of the groups electron donating groups and into the other electron attracting groups.

6. Method according to Claim 2, characterized in that as a compound of formula I 1-oleoyl-2-(4-(3-pyrenyl)-buturoyl)-3-oleoyl-sn-glycerol is used.

7. Method according to Claim 2, characterized in that as a compound of formula I 1,2-di-(4-(3-pyrenyl)-buturoyl)-sn-glycero-sn-3-phosphorylglycerol is used.

8. Compound to be used for carrying out the method according to Claim 1, characterized in that it is an acyl- or an acyl-alkyl-glycerol, or -phosphoglycerol, wherein at least one of the acyl or alkyl groups contains a fluorescent group, and the other groups may optionally contain a fluorescence quenching group.

9. Compound according to Claim 8, c h a r a c - t e r i z e d in that it has the formula

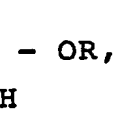


I

wherein

a) at least two of the groups R^1, R^2 and R^3 denote a saturated or unsaturated acylgroup with 3 to 36 carbon atoms, and the third of these can denote also hydrogen or a saturated or unsaturated alkyl group with 3 to 36 carbon atoms, or

b) one of the groups R^2 and R^3 denotes a phosphoryl group



wherein R is hydrogen, ethanolamine, ethylene glycol, choline, glycerol or serine, and the other, as well as R^1, denotes an acyl or alkyl group as defined above, provided, however, that the carbon atom in 2-position to the phosphoryl group may contain only an acyl group, and

wherein in the compound of formula I, always at least one of the acyl or alkyl groups R^1, R^2 and R^3 is substituted with a fluorescent group, and one or both other groups may be optionally substituted with fluorescence quenching group.

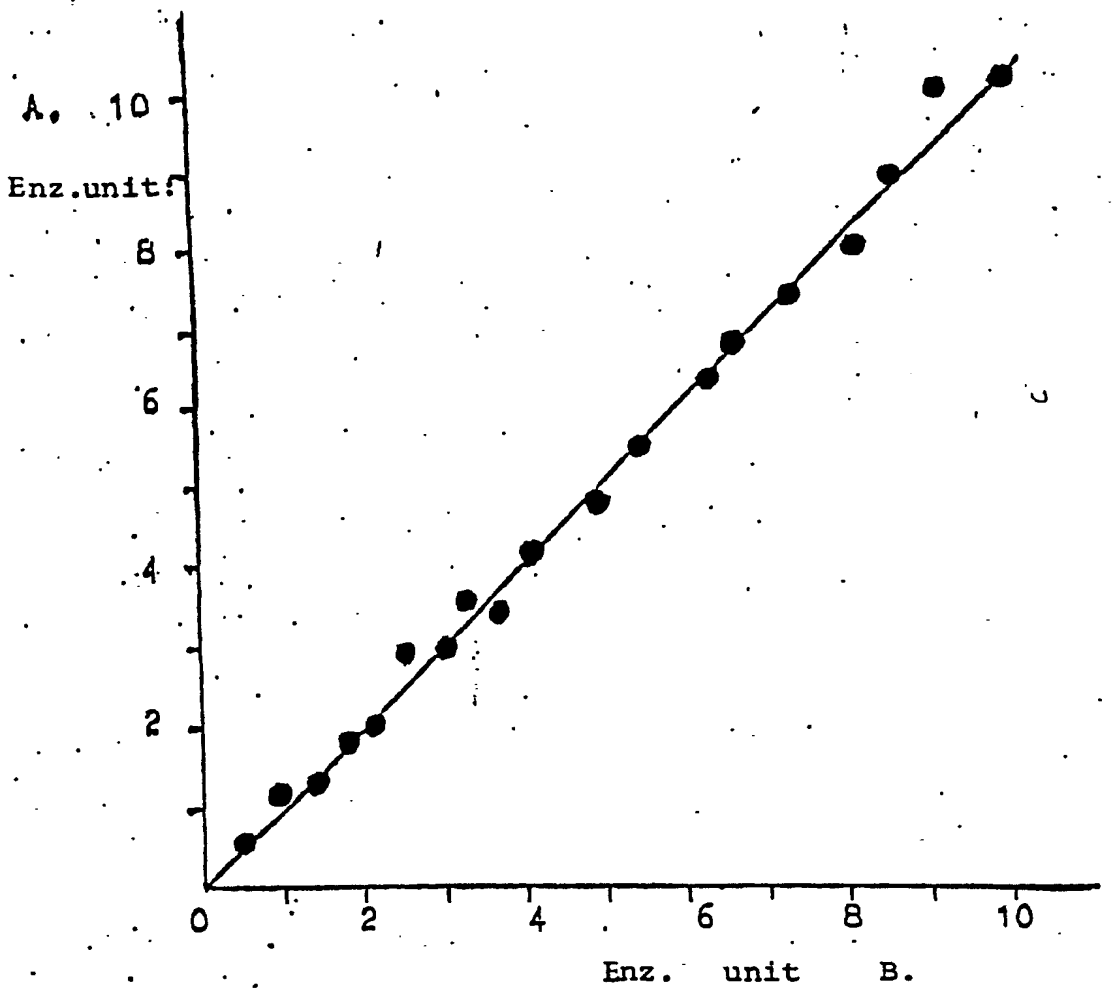
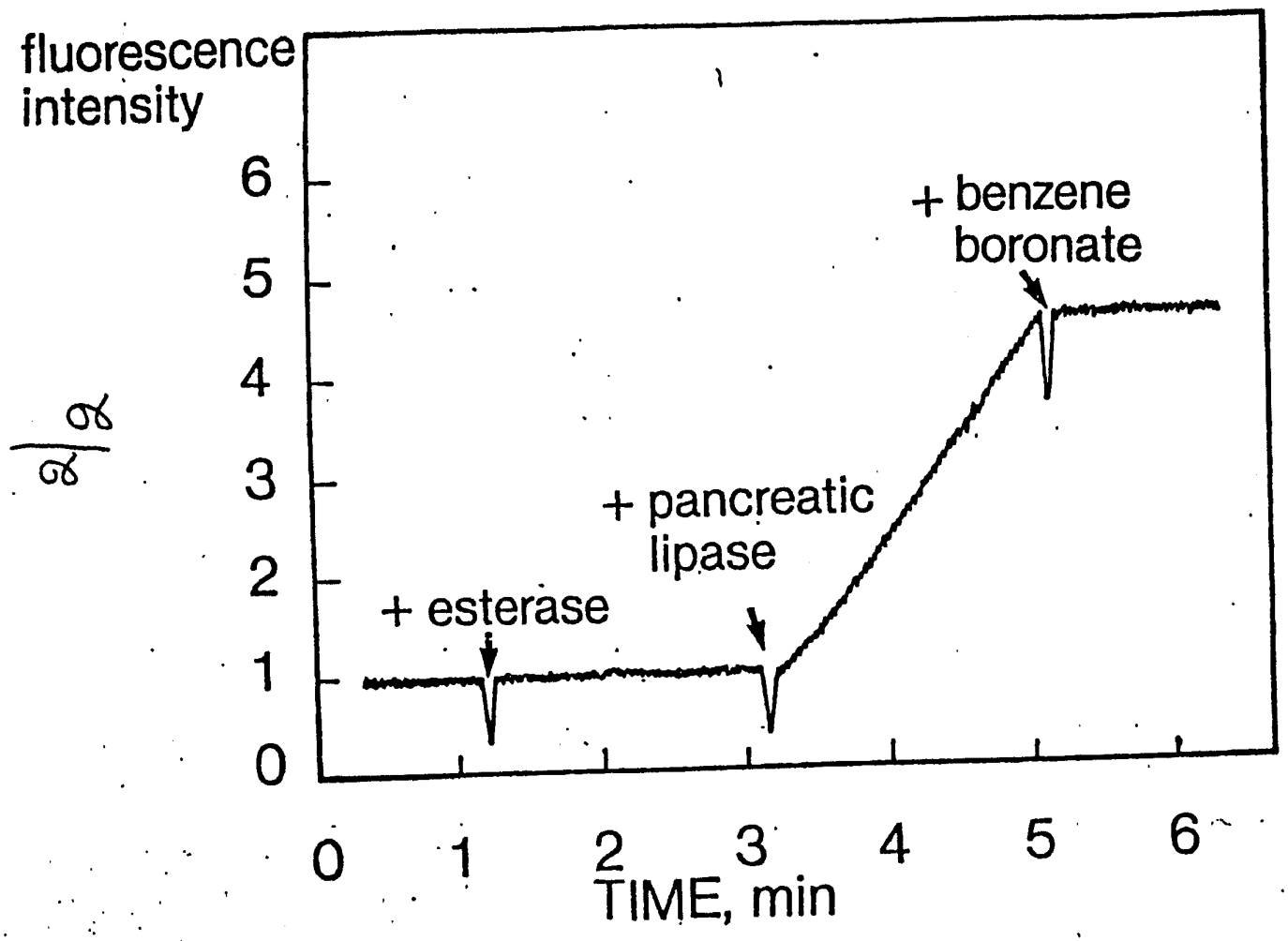


Fig. 1

0037583

Fig. 2

Specifity of the assay



European Patent Office

EUROPEAN SEARCH REPORT

0037583
Application number
EP 81 10 2650

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim
	CHEMICAL ABSTRACTS, vol. 75, no. 17, October 25, 1971, page 27, abstract 105296c, COLUMBUS, OHIO (US) M. FLEISHER et al.: "Automated, fluorometric procedure for determining serum lipase" & Clin. Chem. 17(5), 1971, 417-422 * The whole abstract * ---	1
	CHEMICAL ABSTRACTS, vol. 70, no. 15, April 14, 1969, page 28, abstract 64514y, COLUMBUS, OHIO (US) J.G. MEYER-BERTENRATH et al.: "Properties of new substrates for the determination of pancreas enzymes" & Z. Klin. Chem. Klin. Biochem., 6(6), 1968, 484-488 * The whole abstract * ---	1
A	US - A - 3 986 930 (KUROOKA et al.)	
A	FR - M - 7600 (J. MEYER-BERTENRATH)	
A	US - A - 3 741 876 (G.G. GUILBAULT et al.)	

CLASSIFICATION OF THE APPLICATION (Int. Cl.³)

C 12 Q 1/00
1/34
1/44

TECHNICAL FIELDS SEARCHED (Int. Cl.³)

C 12 Q 1/00
1/34
1/44
G 01 N 33/92

CATEGORY OF CITED DOCUMENTS

X. particularly relevant
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention
E. conflicting application
D: document cited in the application
L: citation for other reasons

& member of the same patent family, corresponding document

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
The Hague	15.07.1981	GRIFFITH